Nutrient Transport and Metabolism in the Life Cycle of Arbuscular Mycorrhizae as Examined by NMR Spectroscopy

INTRODUCTION

Arbuscular mycorrhizal (AM) fungi are obligate symbionts that colonize the roots of the majority of crop plants. Among the wide variety of benefits to the host plant that have been ascribed to this symbiosis are resistance to plant pests (Hooker et al., 1994), improved water relations (Davies et al., 1993), increased growth and yield (Mosse, 1973), and increased nutrient uptake (George et al., 1995). In these associations fungal hyphae grow both in the apoplastic space and also through the host cell walls. Inside host cells the hyphae terminate in swellings called vesicles or highly-branched structures known as arbuscules. These structures do not disrupt the host cell's plasmalemma but penetrate the cell wall and invaginate into the cell so that fungal and host membranes are in close contact. Arbuscules are thought to be involved in phosphorus (Smith, 1995) and carbon transport between the fungus and plant host, while vesicles function as storage compartments for lipids (Cox et al., 1975). Vesicles may not always be present in these associations, and arbuscules have a functional lifetime of 10 to 14 d and may appear only during certain periods of the symbiotic life cycle (Smith and Read, 1997). As with the ectomycorrhizae, hyphae extend out into the surrounding soil and increase the absorption of nutrients such as phosphate and other minerals by the roots. When soil nutrients are in low concentrations the hyphae extend beyond the nutrient-depleted zones and explore greater volumes of soil. Also, since the hyphae are consideraby thinner than roots they can penetrate smaller pores within the soil. The extraradical mycelium of the fungus acts as an extension of the root system,

ıows an experimental design that was used to examine the short-term labeling of ıtact and extracts of leek roots colonized by *Glomus etunicatum* (Shachar-Hill et l., 1995). Excised mycorrhizal roots were perfused with oxygenated buffer as llustrated in Figure 2. The ^{13}C-labeled substrates were added to the perfusion nedium during the experiment or to the medium in which the intact plant was ›reincubated on the bench before excision and examination by NMR. Extracts of he roots were routinely examined to identify and quantify the various labeled and unlabeled metabolites. The advantage of this method is that it yields data on the time course of metabolic events. Since the metabolic fate of the exogenously-supplied substrates is different in each symbiont, one can follow the separate signals from host and fungal compounds in the symbiosis (Shachar-Hill et al. 1995). The disadvantages of this method are that it can be implemented only for short periods of time (up to 48 h) because of possible contamination, and that it is limited to the study of the root and fungal tissues in and on the root (as the extraradical hyphae and spores become detached when the plants are removed from the soil).

In order to follow long-term labeling and the production of sufficient numbers of sterile, viable spores for studying germination processes, sterile growth

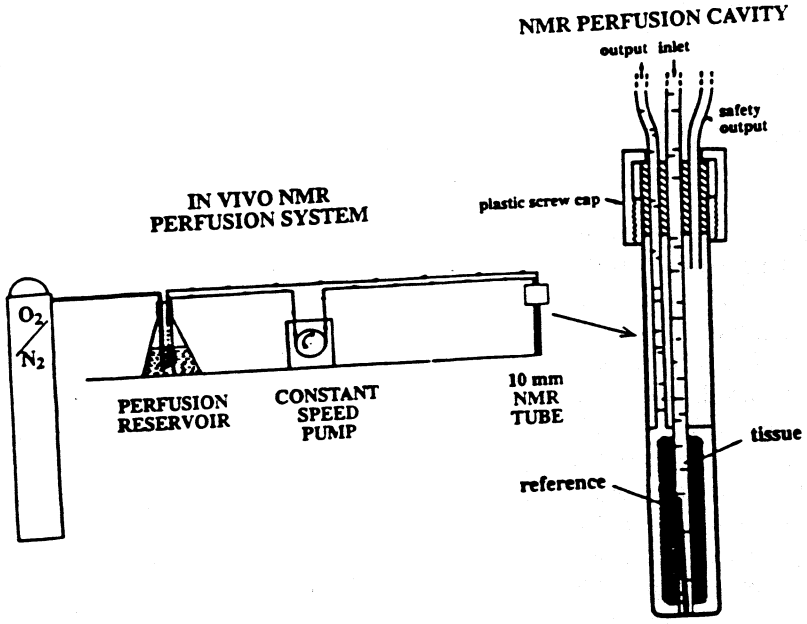

Figure 2. Schematic of perfusion system used for in vivo ^{13}C NMR experiments. Samples were typically perfused with 10 mM Mes buffer solution containing 0.5 mM ^{13}C-labeled carbohydrates (e.g., glucose, fructose, mannitol, etc.).

ity of exploring this transition is enticing because of its importance for understanding the establishment of the AM symbiosis and for its significance in trying to culture AM fungi axenically. To grow axenically, the fungus must be induced to take up sufficient carbon, nitrogen, and other required nutrients from exogenous sources to meet its metabolic requirements fully. Before this can be attempted, however, the metabolism of the fungus both in the functional symbiotic state and the germinating spores needs to be understood better. In this context, the role of host signals in inducing changes in germinating AM fungi is also an area of great interest. This area of investigation has been the subject of research in our group and elsewhere (see review, Bécard et al., 1992b; Giovanetti et al., 1994; Nagahashi and Douds, 1995; Douds et al., 1996; Nagahashi et al., 1996a; Pfeffer and Shachar-Hill, 1996), but will not be covered here. For a recent review the reader is referred to the latest edition of *Mycorrhizal Symbiosis* by Smith and Read (1997).

In this chapter we discuss detailed information we have obtained concerning the biochemistry of AM fungi at different stages of their life cycle (i.e., germination, colonization, and sporulation) using largely stable isotopes in conjunction with NMR spectroscopy and mass spectrometry. For a review of basic NMR and its application in plant biology, see volume 16 of this series (Shachar-Hill and Pfeffer, eds, 1996). This approach can lead to a better understanding of uptake, transport, and intermediary metabolism. In particular we shall cover our recent work on metabolic responses of each symbiotic partner; differential functioning of intra- and extraradical fungal tissues; the location of the synthesis of stored fungal lipids; the contribution of various metabolic pathways to the production of fungal lipids from carbohydrates; hyphal uptake and metabolism of various forms of nitrogen compounds; and metabolic pathways active during germination and sporulation of the fungi.

THE USE OF ISOTOPE LABELING FOR STUDYING TRANSPORT AND METABOLISM

Isotopic labeling has long provided a powerful approach to study biochemical and transport processes in living systems. Applying these methods to studying AM systems is difficult, however, for a number of reasons: the presence of two organisms (of which the fungal symbiont cannot be grown or studied separately); the small volume occupied by the fungus in the symbiosis, and the fact that it cannot be separated intact from the host; the presence of other microorganisms on and around most mycorrhizal plants; and the difficulty of providing label selectively to one partner, particularly for nutrients that are freely mobile in the soil. These factors limit the ease with which the organisms, their products, and their transport mechanisms can be separated, a process inherent in most analyses. The use of analytical tools that do not require chemical or physical separation of metabolites are thus particularly advantageous here, and NMR spectroscopy is among the most informative of such methods. The two experimental systems illustrated in Figure 1 reduce or circumvent the above difficulties. Figure 1A

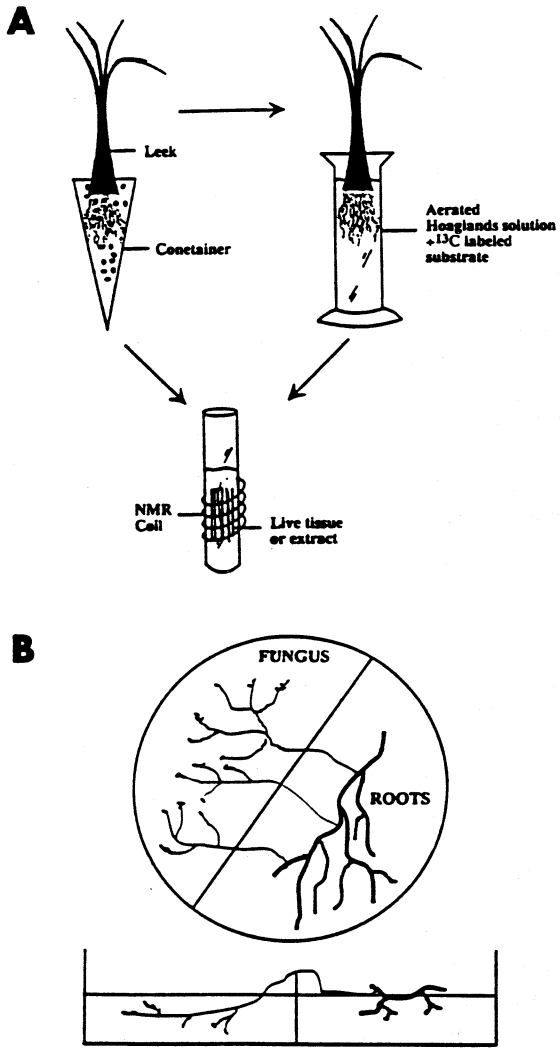

Figure 1. Spectra were obtained on both whole prelabeled and unlabeled tissue perfused with ^{13}C substrates as well as extracts. A, Schematic of labeling procedure for examining colonized and uncolonized leek plants using ^{13}C-labeled substrates. Following the crossover of hyphae, labeled substrates were added to either compartment. After four weeks the two compartments containing the roots and fungal material (external hyphae and daughter spores) were separated and harvested using citrate to solubilize the gellan for easy removal of the tissue. To examine the water-soluble and lipid-soluble compounds, the tissues were extracted with 70:30 methanol:H_2O and isopropyl alcohol, respectively. B, Schematic of sterile dual-culture divided–Petri plate design for long-term labeling, adapted from St.-Arnaud et al., 1996.

ıows an experimental design that was used to examine the short-term labeling of ıtact and extracts of leek roots colonized by *Glomus etunicatum* (Shachar-Hill et l., 1995). Excised mycorrhizal roots were perfused with oxygenated buffer as llustrated in Figure 2. The ^{13}C-labeled substrates were added to the perfusion nedium during the experiment or to the medium in which the intact plant was ›reincubated on the bench before excision and examination by NMR. Extracts of he roots were routinely examined to identify and quantify the various labeled and unlabeled metabolites. The advantage of this method is that it yields data on the time course of metabolic events. Since the metabolic fate of the exogenously-supplied substrates is different in each symbiont, one can follow the separate signals from host and fungal compounds in the symbiosis (Shachar-Hill et al. 1995). The disadvantages of this method are that it can be implemented only for short periods of time (up to 48 h) because of possible contamination, and that it is limited to the study of the root and fungal tissues in and on the root (as the extraradical hyphae and spores become detached when the plants are removed from the soil).

In order to follow long-term labeling and the production of sufficient numbers of sterile, viable spores for studying germination processes, sterile growth

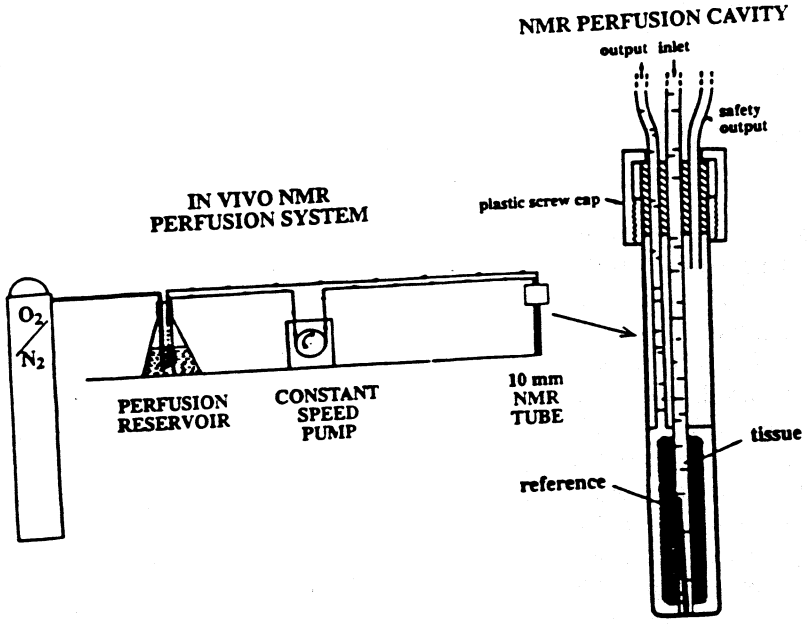

Figure 2. Schematic of perfusion system used for in vivo ^{13}C NMR experiments. Samples were typically perfused with 10 mM Mes buffer solution containing 0.5 mM ^{13}C-labeled carbohydrates (e.g., glucose, fructose, mannitol, etc.).

conditions are required. Figure 1B illustrates the experimental design reported by St.-Arnaud et al. (1996). In this system, Ri T-DNA–transformed carrot (*Daucus carota* L.) roots are grown in sterile divided plates containing solidified minimal M medium (Bécard and Fortin, 1988). Roots colonized by *Glomus intraradices* are grown in one compartment until the mycelium has crossed over the central barrier to the unoccupied compartment before the formation of many spores. This system allows the study of transport and metabolism both within the root and in the extraradical hyphae and spores. An isotopically-labeled substrate is added as a sterile solution to one side of the divided plate and the plates are incubated for another four to eight weeks. During this time the fungus grows and sporulates extensively in the fungal compartment so that almost all the tissue in the fungal compartment is formed during the labeling period. The colonized roots and fungal hyphae and spores from each compartment are then isolated separately from the solidified medium under mild conditions (Doner and Bécard, 1991). The fungal compartment is also an excellent source of large quantities of sterile, viable spores that are used for studying germination processes (see below). The tissue from each compartment is lyophilized and extracted with methanol-water and subsequently with isopropanol. A series of such experiments with associated analyses and controls (including identifying labeled compounds in both the host and the fungus, determinations of the effects of unlabeled substrates on growth in the system, and labeling in uncolonized roots) are carried out as well. The fractional labeling at different carbon positions of the major fungal and plant products of these compounds is subsequently determined by NMR and GC-MS.

SHORT-TERM ^{13}C-LABELING STUDIES OF LEEKS COLONIZED BY *G. ETUNICATUM*

Substantial amounts of fixed carbon are transferred from the host plant to the fungal symbiont, but despite the magnitude of this transfer, the details of carbon transport and metabolism by AM fungi are not well understood. The AM fungi occupy small volume fractions in mycorrhizal root tissues (Smith and Read, 1997), and neither dissection nor axenic culturing has yielded sufficient quantities of fungal material for detailed metabolic study (Bécard and Fortin, 1988). These difficulties have meant that little detail is known of the metabolic routes by which carbon fixed by the host is utilized by the fungus, and still less is known of the effects (if any) of the host on fungal metabolism. Studies by Lösel and Cooper (1978) and Bevege et al. (1975) using ^{14}C radiolabeling give information on the classes of compounds labeled in AM colonized tissue by photosynthate and various root-fed substrates, but do not elucidate the labeled structures or possible pathways that may produce them. Much more detailed metabolic information is available from studies of axenically-cultured ectomycorrhizal fungi (Ramstedt et al., 1987; Martin et al., 1988). In these and in some nonmycorrhizal fungi (Jennings and Burke, 1990) mannitol becomes highly labeled when labeled glucose is supplied and probably plays important if still disputed role(s) (Martin

al., 1988; Jennings and Burke, 1990). These studies have also revealed metabolite cycling, as reflected in the substantial fractions of label supplied as glucose which are scrambled among different positions of hexose and hexitol molecules. It is known that AM fungal spores contain trehalose as their major carbohydrate store (Bécard et al., 1992a; Schubert et al., 1992) and minor amounts of trehalose also have been reported in extraradical mycelium (Amijee and Stribley, 1987) and mycorrhizal roots (Schubert et al., 1992). The extent to which the carbohydrate metabolism of AM is otherwise different than that of ectomycorrhizal species was not known, however, until it was revealed recently by in vivo NMR experiments (Shachar-Hill et al., 1995).

Using the methodology depicted in Figure 1A, colonized and uncolonized leek roots were perfused with 0.5 mM ^{13}C–1 glucose to assess carbohydrate partitioning. In vivo natural-abundance NMR time-course spectra (3 h each) of excised uncolonized leeks showed resonances attributed to sucrose C1, C2', and C2 of fructan polymers (see Fig. 3 legend). Upon perfusion with 0.5 mM ^{13}C–1-labeled glucose, little change in the spectum was observed except for the labeling of C-1 sucrose following the washout of the perfusing labeled glucose. Little change in the perfusing α and β-glucose levels were observed, even after 18 h of circulation. In contrast, the leek roots colonized by *G. etunicatum* showed a significant depletion of the circulating glucose, as well as significant labeling of fungal carbohydrates (trehalose and glycogen) and no labeling of host sucrose over 24 h. Similar results were observed when roots of whole plants were prelabeled with 50 mM ^{13}C-1 glucose for 20 h, then excised and examined by in vivo NMR. That colonization reduced the level of labeled sucrose by the host root cells even at high and low glucose levels indicates that regulation of host metabolism rather than competition may be at work. Since fractional enrichment of ^{13}C in trehalose was greater than 72%, little dilution of externally-supplied labeled sugars by host photosynthate during synthesis was indicated. These data are consistent with glucose as a major form of fixed carbon taken up directly from the apoplast by the fungus; however, the mechanism and location of the transporter is yet unknown. A recent study has shown that a plant hexose transporter is induced in the plant cortical cells during symbiosis, but it is an uptake transporter; therefore it is unclear whether it is involved in the efflux of carbon (Harrison, 1996). More recently, using radiorespirometry Solaiman and Saito (1997) have confirmed our findings (Shachar-Hill et al., 1995) that glucose is the main substrate used by the internal hyphae (Saito, 1995, 1997) of mycorrhizal roots.

Koide (1991) suggested that application of phosphate might simulate the metabolic effects of mycorrhizae on host plants. To test this theory, the glucose labeling experiments were carried out with phosphate as a substitute for mycorrhizal colonization. Under these conditions sucrose labeling was the same as in the uncolonized control, indicating that phosphate does not mediate the host plant metabolism in the same manner as mycorrhizal infection.

In contrast to free-living ectomycorrhizal fungi (Martin et al., 1985) the arbuscular mycorrhizae convert glucose directly to trehalose without cycling

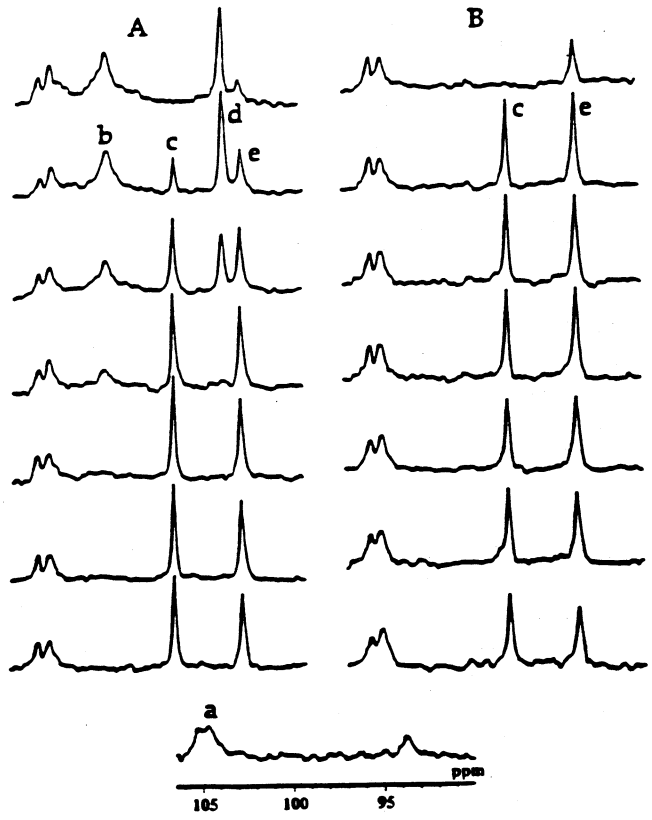

Figure 3. Time courses of in vivo ^{13}C NMR spectra of colonized (A) and uncolonized (B) leek roots excised and perfused with 0.6 mM $^{13}C_1$ glucose in Hoagland's solution. Each successive spectrum was acquired in 3 h. The first spectrum (at the bottom of the figure) was acquired before the addition of the $^{13}C_1$ glucose to the perfusion medium and was essentially identical for both mycorrhizal and control roots. Labeling of peaks: a, C_2 of sucrose and C_2 of fructans; b, C_1 of glycogen; c, C_1 of β-glucose; d, $C_1$of trehalose; e, C_1 of α-glucose and C_1 of sucrose. Last spectrum in each time course was acquired after washout with glucose-free solution after 24 h of exposure to glucose. Adapted from Shachar-Hill, 1995.

through mannitol, since no labeled mannitol or ^{13}C-1,C-6-labeled trehalose is observed in AM fungi (Shachar-Hill et al., 1995). It has been suggested (Ramstedt et al., 1987; Martin et al., 1988) that this cycling or the net synthesis rate of mannitol regulates growth by affecting the NADP/NADPH redox couple. The above findings indicate that the role of mannitol in rapidly-growing, free-living fungi may be different from that in the symbiotic state. Experiments also

lemonstrated that the lag time (12–15 h) for the formation of the fungal netabolites may be a function of the availability of photosynthate, since this time vas considerably shortened when photosynthate was low in the host (data not hown). In addition, it was noted that the production of trehalose and glycogen ccurred only after a very long lag (greater than 36 h) in the colonized host tissue iat was devoid of arbuscules, indicating that arbuscule formation is in some way orrelated with the production of these storage compounds (Pfeffer et al., 1996; feffer and Shachar-Hill, 1996). One possibility for the activation of this synthesis hen arbuscules appear is that the presporulation phase of the fungal life cycle has egun, since spores contain stores of these compounds prior to germination Bécard et al., 1992a).

LONG-TERM ^{13}C-LABELING STUDIES OF CARBON UPTAKE AND METABOLISM IN AM FUNGI

Long-term labeling experiments using the divided Petri plates with transrmed carrot roots (see above and Fig. 1B) make it possible to determine the ecific sites of uptake and metabolism of a range of labeled substrates. Under ng-term labeling conditions only minor amounts of the labeled fungal metaboes, trehalose and glycogen, are observed, but more label is incorporated into ids. Labeling in plant and fungal lipids gives valuable information regarding tabolic pathways involved in long-term storage.

Labeling experiments were carried out by placing various ^{13}C-labeled subates in either of the two compartments of the Petri plate containing colonized ot tissue or the compartment with hyphae and developing spores (unpublished a). *G. intraradices* produces predominantly one stored fatty acid, namely .12-hexadecenoic acid (ω-5 palmitic acid), under all growth conditions (Gran et al., 1995). This fatty acid is not made by the host, so it is possible to tinguish the plant lipids in crude extracts of colonized roots dominated by 0,12,13 octadecadienoic acid (linoleic acid) from the fungal lipid products in colonized root tissue. By examining extracts of the colonized root tissue taken n the Petri plate after incubation with ^{13}C-1 glucose we observed labeled fungal ds in the presence of the labeled host lipids. Figure 4 shows parts of three ^{13}C ctra of the double-bond region of carrot tissue extracts in both colonized and olonized states. Figure 4A displays the four double-bond resonances of the olonized tissue without previous labeling. Note that all four resonances had al intensity, representing the background or natural abundance levels of ^{13}C %). Spectra of extracts of uncolonized tissue labeled with ^{13}C-1 glucose wed that the even-numbered double-bond carbon resonances (C-10 and C-12) approximately three times the intensity of the odd-numbered carbon resoes (C-9 and C-13; Fig. 4B) due to labeling in these positions. This result is sistent with the catabolism of ^{13}C-1 glucose via glycolysis to produce ^{13}C-2 yl CoA, which then incorporates a ^{13}C labeled at every other even carbon along inoeate chain. The result of an experiment carried out with the colonized roots

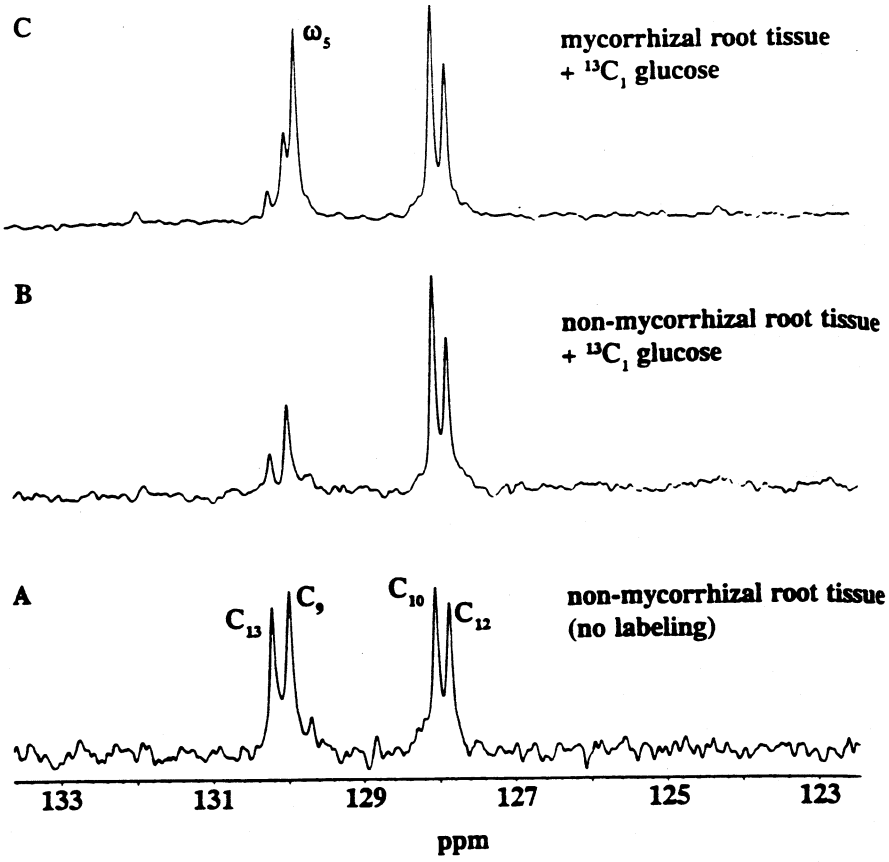

Figure 4. ^{13}C NMR spectra of the double-bond region of isopropyl alcohol extracts of *G. intraradices* colonized and noncolonized transformed carrot roots. A, Extract of non-mycorrhizal root. B, Extract of non-mycorrhizal tissue incubated in the Petri plate with 30 mM ^{13}C-1 glucose for four weeks. C, Extract of mycorrhizal tissue incubated in the Petri plate with 30 mM ^{13}C-1 glucose for four weeks. Each extract was obtained from the root compartment of the divided plate. The spectra were obtained in 8 h.

is shown in Figure 4C. Labeling of linoleate (C-10, C-12) at 127.7 and 127.9 ppm and C-12 of the ω-5 palmitic acid was observed at 129.9 ppm. More information regarding the uptake and metabolism of various substrates was available from the rest of the spectrum. For example, Figure 5B shows a spectrum of an extract of colonized carrot root tissue labeled in the root compartment with ^{13}C-1 glucose. The same double-bond resonance pattern appears in Figure 4C; however, one may also see that the terminal carbon resonance (even-numbered carbon) at 14.1 ppm

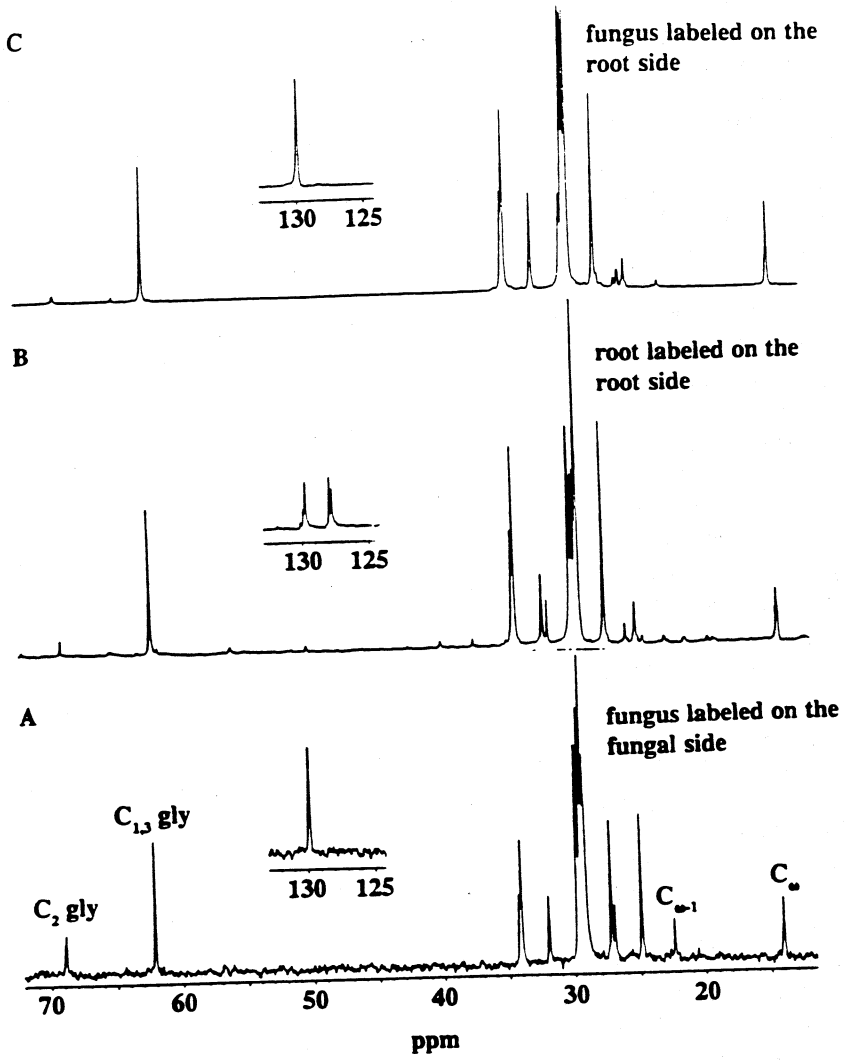

Figure 5. ^{13}C NMR spectra of isopropyl alcohol extracts of root and fungal tissue taken from the divided Petri plate following incubation for four weeks with 30 mM ^{13}C-1 glucose on either side. A, Spectrum of the extract of fungal tissue labeled in the fungal compartment. B, Spectrum of the extract of the root tissue labeled in the root compartment. C, Spectrum of the extract of the fungal tissue labeled in the root compartment.

was significantly greater than the signal from the penultimate carbon resonance (odd-numbered carbon) at 22.3 ppm. This result is consistent with what one would expect to observe in the synthesis of the fatty acid chain from labeled acetyl Co

A. Likewise, the signal from C-1 and C-3 of glycerol (carbon 1 and carbon 3 are equivalent and therefore share the same resonance position) is more than twice as large as the C-2 signal of the glycerol moiety of the triacylgercerol (triglycerides). This labeling pattern is produced through the breakdown of glucose to triose, which can carry the ^{13}C label only into the 1,3 positions of glycerol. From such data it is calculated that the fractional enrichment in the lipids (plant and fungal) is 12.4% and that uptake and metabolism of ^{13}C-1 glucose is active within and around the root tissue (unpublished data). Similar labeling profiles were obtained for the fungal tissue (hyphae and spores) from the fungal compartment after the root tissue in the other compartment was exposed to ^{13}C-1 glucose (Fig. 5C). This lipid, which is solely of fungal origin (primarily ω-5 palmitate as evidenced by the single double-bond resonance at 129.9 ppm), was labeled to the extent of 21.0%. This higher labeling level in the fungal lipids as compared with the root tissue is to be expected, since the lipid in the fungal compartment is mainly newly synthesized after label was added, while the root contains stores of unlabeled lipids formed before addition of ^{13}C-1 glucose. In contrast to the above experiments, incubation with ^{13}C-1 glucose in the fungal compartment produced fungal tissues whose spectra showed no observable labeling (see Fig. 5A). The same result was obtained in the analysis of the corresponding root lipids, so no uptake and metabolism of glucose was evident through the external hyphae (data not shown). Similar results were obtained using ^{13}C-1 fructose. Labeling the root compartment with ^{13}C-2 acetate, however, produced labeled organic acids in addition to labeled lipids within the colonized root.

To be certain that isotope incorporation was not being observed in the fungal compartment because of the dominance of a pentose phosphate pathway (within the fungus) in which the C-1 of glucose or fructose was lost, the fungal compartment was incubated with ^{13}C-1,2 glucose. No labeling was observed when this was added to the fungal compartment, showing that the lack of observed labeling when ^{13}C-1 glucose was used was not due to an active pentose phosphate pathway. Peak splitting seen in signals of double labeled molecules obtained from metabolite labeling with ^{13}C-1,2 glucose allow us to distinguish between products derived through glycolytic and pentose phosphate pathways. When ^{13}C-1,2 glucose was added to the root compartment it was revealed that approximately 80% of the fungal lipids were produced through the glycolytic pathway and approximately 20% through the pentose phosphate pathway.

DETERMINATION OF THE LOCATION OF LIPID SYNTHESIS IN ARBUSCULAR MYCORRHIZAE

While the results described above demonstrate that, unlike the internal fungal hyphae, external hyphae are not capable of taking up carbohydrates, these experiments cannot pinpoint where lipid is synthesized. The question remains whether the carbohydrate is transported to the spores and there converted into lipid, or whether carbohydrates are first converted into lipids and then transported out via hyphae to the developing spores.

Using the dual-culture system of colonized carrot roots described above, ·-labeled acetate was supplied to either the root or fungal compartment. Our ɔeriments to date show that acetate supplied to the root compartment resulted ¹³C labeling of both lipids and organic acids in the colonized roots but not in the ıgal compartment, whereas no lipid labeling was observed in either compart-ːnt when ^{13}C-2 acetate was supplied to the fungal compartment (in contrast to observation that acetate supplied to germinating spores resulted in rather ective labeling of trehalose; see below). These results did not establish the site lipid synthesis, since in no case were lipids labeled in the fungal compartment acetate.

Because water is freely permeable across cell membranes, and the synthesis fatty acids results in the incorporation of hydrogens that are exchangeable with ıter into nonexchangeable positions bonded to carbon atoms, water with ɔtopically-labeled hydrogens is a suitable marker for lipid biosynthesis. We ːrefore used deuterated water (^2H_2O) added to either compartment at a final ncentration of 2 or 3% (levels which do not slow growth) and analyzed the uteration levels in lipids in each compartment. At low levels of isotopic richment (necessary because high levels of deuterated water inhibit growth), ^{2}H ⁄IR proved a more straightforward method than GC-MS for detecting labeling lipids. Because of evaporative exchange of water between compartments, uterium labeling incubations were carried out for only two weeks. The labeling fungal lipids in the fungal compartment was much higher when ^2H_2O was ɔplied to the root compartment; indeed, the level of labeling in fungal compart-ːnt lipids when ^2H_2O was supplied to the fungal compartment was of the level ɔwn in the control (without roots present), which is due to evaporative exchange which ^2H_2O may enter the root compartment. Thus, we conclude that most or lipid stored in the fungal spores is synthesized in the root compartment. This ding seems to be the only direct evidence to support the hypothesis first put ·ward by Cox et al. (1975) concerning the site of lipid synthesis. These ːearchers made microscopic observations on lipid droplets in hyphae and ɔuscules of *Allium cepa* L. colonized by *Glomus mosseae* and concluded that ːse droplets were probably synthesized by the fungus in the host roots and nsported from there.

^{15}N-LABELING EXPERIMENTS

The importance of vesicular-arbuscular mycorrhizal (VAM) fungi to plants enhancing phosphorous uptake has been established in many studies, but their nificance in nitrogen nutrition seems uncertain. Tawarays and Saito (1994) ve shown that there is no change in amino acid composition in mycorrhizal ıon and white clover roots relative to uncolonized roots. A number of research- have demonstrated that external hyphae of VAM fungi can take up and ·tabolize nitrate (Bago et al., 1996). Others have used ^{15}N-labeled ammonia and rate to show that nitrogen can be taken up from the soil by VAM fungal hyphae ɔ transferred to the host (Johansen et al., 1996). In contrast, other studies have

shown no enhancement of nitrogen label uptake in mycorrhizal compared to non-mycorrhizal plants or have raised doubts about nitrate uptake by the fungus (Villegas et al., 1996; for a recent review of the literature see Smith and Read, 1997). Most significantly, there is little definitive evidence of a nutritional benefit to the host due to the transfer of nitrogen from the fungus. Since nitrogen is usually found as nitrate, which is much more mobile in the soil than phosphorous, increased exploration of the soil by VAM fungal hyphae may not significantly enhance nitrogen uptake. Indeed, evidence to this effect was obtained by Tobar et al. (1994), who showed that VAM colonization improved the uptake of nitrogen label by *Glomus fasciculatum* only under dry conditions, in which nitrate mobility is lower.

We have used ^{15}N-labeled substrates in the in vitro carrot root and colonized leek systems together with extraction and GC-MS analysis of amino acids to compare quantitatively the forms of nitrogen taken up by each partner and transferred to the other. The use of the split plate system (Fig. 1B) allows us to compare uptake and transfer of label supplied in different forms without the confounding effects of diffusion between compartments and dilution and interconversion of nitrogen forms that occur in experiments using plants growing in nonsterile soil. Our preliminary results indicate that during a six-week labeled culture period, the external hyphae of *G. intraradices* are able effectively to take up ^{15}N label supplied as ammonium, nitrate, or urea, but not as glycine. ^{15}N-label levels in the free-amino acid pools of host roots in the root compartment were highly labeled from label supplied to the fungal compartment. The levels of labeling in different amino acids (30–80%) of the host roots are substantially higher than those reported in previous studies. The levels of labeling in host amino acids were similar whether label was supplied to the host or fungal compartments, showing that the fungus gives the host free access to nitrogen in the fungal compartment. This study appears to be the first in which an organic form of nitrogen (urea) is shown to be made available to the host by the fungal symbiont. This finding and the rather high labeling levels found in host amino acid pools shows that the fungus can be an effective conduit for movement of nitrogen from the environment to the host.

There is a striking contrast between the complete lack of carbon label taken up by the external fungal structures and the rather effective uptake of different nitrogen forms in these experiments. In the case of leek plants the ^{15}N-labeling levels in the free amino acids were compared between leaves and roots of colonized and two groups of uncolonized plants (at low and high phosphorous fertilization) after fertilization with ^{15}N nitrate for several weeks. In this case, colonization did not affect the relative or absolute labeling levels in different amino acids of leaf or root tissues, despite the fact that colonization levels were high (greater than 40%) and the colonized plants grew better than uncolonized ones at low phosphorous levels. There was no evidence for either changes in steady-state label fluxes nor for transfer of significant amounts of nitrogen from fungus to host. In this system the plants were well watered and there was no

ompartmentation of the soil (see Fig. 1A) so that the roots explored the soil oroughly, whether colonized or not. Taken together, the results of these studies dicate that at least some VAM fungi are able to take up different forms of itrogen very effectively and to transfer them to the host roots, but that this ability ay not be significant for nitrogen metabolism, either in a regulatory or direct utritional sense, in situations in which nitrogen is either abundant or mobile or oth.

STUDIES OF SPORE GERMINATION

Spore germination entails the mobilization of stored polyphosphate and the reakdown of stored lipids and carbohydrates. We used ^{31}P to monitor the ermination process in AM spores. *Gigaspora margarita* spores were incubated t 30°C for 5 d and ^{31}P spectra taken each day. Initially only PolyP signals were etected, indicating an average chain length of 11 units. As the spores began to erminate, narrow signals associated with newly-formed Pi, UDPG, NADP(H), nd phosphomonesters were seen, as well as a broad resonance (extending from 0 to 20 ppm) from relatively immobile, phospholipid membranes.

The main storage sugar in the majority of mycorrhizal fungal spores is rehalose (Bécard et al., 1992a). That stored trehalose is utilized in the early stages f germination was revealed by in vivo ^{13}C NMR time-course experiments, which emonstrated a 50 to 58% breakdown over a period of 5 d after germ tube mergence. No significant reduction of lipid signals from triacylglycerols and free atty acids was detected during this time, indicating that lipid stores are probably letabolized to sustain hyphal growth only after the carbohydrate has been xhausted. This observation has recently been supported in a study of lipid omposition in *Glomus versiforme* spores following germination (Gaspar et al., 994). The study found that although the total amount of mobile lipids was little hanged in the first 5 d of germination, its composition changed with the ecreasing ratio of saturated to unsaturated fatty acid chains. Carbon dioxide has stimulatory affect on the growth of emerging hyphae (Bécard and Piché, 1989; Bécard et al., 1992b). Nothing is known about the fate of this CO_2, but it has been ypothesized that the spore could be utilizing it in dark fixation (Bécard and Piché, 989).

To examine the metabolic events associated with carbohydrate utilization uring germination, sterile *G. intraradices* spores isolated from the dual-culture ivided-plate system described above (Fig. 1B) were germinated for 12 d with abeled substrates (^{13}C-1 glucose, ^{13}C-2 acetate, ^{13}C-1 fructose, ^{13}C-1,6 mannitol r $^{13}CO_2$). The ^{13}C NMR spectra of lipid extracts showed no evidence of fatty acid abeling, although low levels of labeling could have been obscured by large mounts of unlabeled lipid present before germination. Labeling patterns in rehalose from aqueous extracts of ^{13}C-1 glucose– and ^{13}C-1 fructose–treated amples showed, however, that trehalose is labeled in the C-1 position, a onsequence of direct coupling of these monosaccharides (see Fig. 6A). No

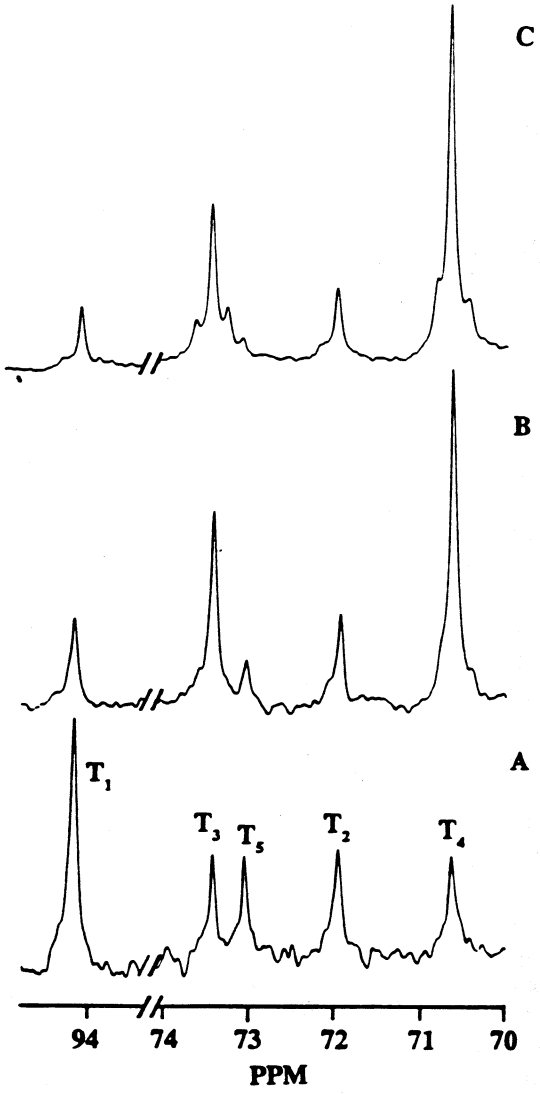

Figure 6. ^{13}C NMR spectra of 70:30 methanol:H_2O extracts of *G. intraradices* spores following germination for 12 d in the presence of ^{13}C–1 glucose (A), $^{13}CO_2$ (B), and ^{13}C–2 acetate (C) in distilled water. The spores were isolated in a sterile state from the in vitro dual-culture system described in Figure 1B.

idence of scrambling of the label into the C-6 position of trehalose (a result of annitol cycling) or other pathways was observed (data not shown). Nor did cubation with ^{13}C-1,6 mannitol produce any labeled trehalose, a further indica- n of the absence of mannitol metabolism in germinating AM spores as it is found ectomycorrhizal fungi (Martin et al., 1985). Following the labeling of the spores ith either $^{13}CO_2$ or ^{13}C-2 acetate, trehalose signals showed labeling predomi- ntly in the C-3 and C-4 positions with less in the 1,2 and 6 positions, spectively. Carbon 5 appeared not to be labeled at all (see Figs. 6B and 6C). It evident from these results that dark fixation of CO_2 is active in germinating ores, and that, unlike the symbiotic state, the gluconeogenic pathway is a major ute to the production of carbohydrate from lipid precursors. It remains to be tablished in what way the metabolism of the germinating spores will be altered hen they are exposed to plant root exudates, which effect the morphology of the tending germ tube and hyphae (Nagahashi et al., 1996b).

SUMMARY OF NEW FINDINGS REGARDING ARBUSCULAR MYCORRHIZAE

In leeks colonized by *G. etunicatum* a hexose (most likely glucose) is taken directly by internal hyphae or arbuscules (Shachar-Hill et al., 1995). This take is influenced principally by the availability of host photosynthate. The ngus utilizes glucose during the production and turnover of arbuscules to oduce trehalose and glycogen. The metabolic rate of glucose utilization in the M tissue during this time is elevated relative to uncolonized plant roots. The esence of the mycorrhizal fungi within the root suppresses the production of host crose, but phosphate was found not to mimic the affect of AM fungi on host crose production.

Figure 7 is a schematic representation of the findings based on the divided–tri plate experiments described above with transformed carrot roots colonized ith *G. intraradices*. Phosphate (P) and nitrogen (N) enter through the extraradical phae and are transferred to the plant cell through the arbuscule. Carbon mpounds (C) such as carbohydrates cannot be taken up by external fungal uctures such as hyphae or developing daughter spores, but enter the fungal uctures within the root apoplast. Carbon is then moved from the intraradical phae in the form of fungal lipids along the hyphae to the developing daughter ores. There appears to be little or no synthesis of fungal lipids in the extraradical sues. In contrast to extraradical fungal tissues and developing daughter spores, rminating fungal spores are capable of taking up various forms of C, including ucose, fructose, acetate, and CO_2. The first two of these substrates are converted rectly to trehalose, whereas acetate is converted via gluconeogenesis to treha- se, and CO_2 undergoes dark fixation to produce trehalose as well. Under normal owing conditions, colonization by mycorrhizal fungi does not appear to enhance uptake of nitrate or ammonium by the roots, but extraradical hyphae are pable of utilizing nitrogen as urea.

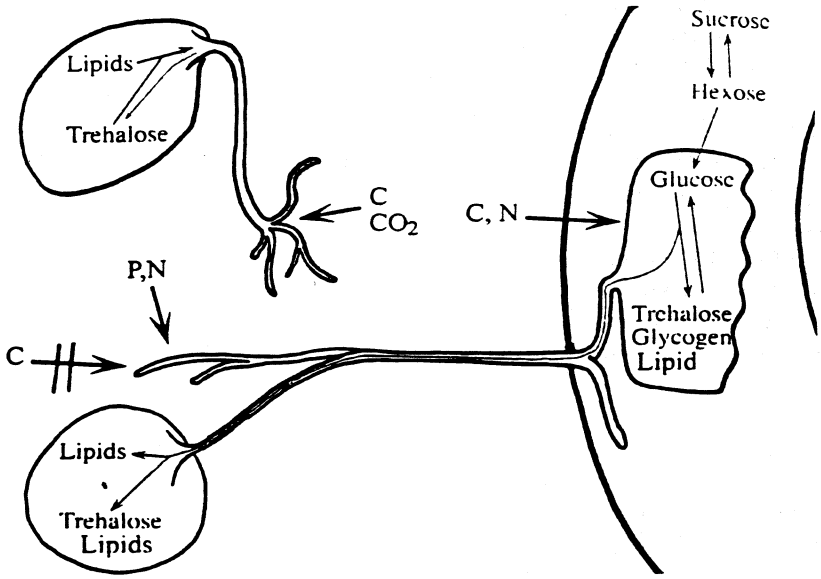

Figure 7. Schematic summary of the uptake, metabolism, and transport of nutrients in mycorrhizal plant roots and extraradical hyphae and spores.

FUTURE DIRECTIONS

The observations on transport and metabolism described above are informative as to the substrates, pathways, and storage products of intermediary carbon metabolism, and are beginning to yield similar information concerning nitrogen uptake and transport. However, our knowledge is still incomplete and further experiments of the sort described above, especially using doubly-labeled substrates, are needed to define quantitatively the relative activities of different pathways at different stages of the life cycle.

One potentially interesting application of findings to date is as a guide to molecular genetic work, first to find the genes involved in the uptake and metabolic processes identified, and then to use these as markers to follow the expression of these genes throughout the fungal life cycle.

Another area of interest concerns the sites and mechanisms of carbon exchange between host and fungus. Preliminary results described above concerning the correlation of hexose uptake with the presence of arbuscules may be related to this isssue, and further such experiments under different growth conditions may be informative. However, the definitive answers on transport location can come only from molecular genetic and immuno-histochemical methods to locate specific transporters, as has been done by Harrison for plant hexose and fungal

osphate transporters (Harrison and van Buuren, 1995; Harrison, 1996) and by aninazzi-Pearson and coworkers (1991) for H^+-ATPase. Such work is techni-lly demanding but important in addressing rigorously the sites and detailed chanisms of transport processes.

Evaluating the metabolic responses of the germinating spores and developing phae to the exuded plant signals is another potentially important area. Metabolic eling studies of this by the methods described above and supported by following gal gene markers should provide insight into the transition from free-living to biotic state. ^{13}C isotopic labeling and NMR and mass spectrometry can also tentially be used in combination with in vitro cultures to track signaling npounds in plant and fungal exudates. Exudates from prelabeled plant and gal tissues could be collected and analyzed to identify specific signal–ognition compounds.

LITERATURE CITED

en MF, Sexton JC, Moore Jr TS, Christensen M (1981) Influence of phosphate source on vesicular-arbuscular mycorrhizae of *Bouteloua gracilis*. New Phytol **87**: 687–694

lijee F, Stribley DP (1987) Soluble carbohydrates of vesicular-arbuscular mycorrhizal fungi. Mycologist **1**: 21–22

jo B, Vierheilig H, Piché Y, Azcón-Aguilar C (1996) Nitrate depletion and pH changes induced by the external mycelium of the arbuscular mycorrhizal fungus *Glomus intraradices* grown in monoxenic culture. New Phytol **133**: 273–280

ard G, Doner LW, Rolin DB, Douds Jr DD, Pfeffer PE (1992a) Identification and quantification of trehalose in vesicular-arbuscular mycorrhizal fungi by in vivo ^{13}C NMR and HPLC analyses. New Phytol **118**: 547–552

ard G, Douds Jr DD, Pfeffer PE (1992b) Extensive in vitro hyphal growth of vesicular-arbuscular mycorrhizal fungi in the presence of CO_2 and flavonols. Appl Envir Microbiol **58**: 821–825

ard G, Fortin JA (1988) Early events of vesicular-arbuscular mycorrhiza formation on Ri T-DNA transformed roots. New Phytol **108**: 211–218

ard G, Piché Y (1989) Fungal growth stimulation by CO_2 and root exudates in vesicular-arbuscular mycorrhizal symbiosis. Applied and Environ Microbiol **55**: 2320–2325

lenfalvay GJ, Schüepp H (1994) Arbuscular mycorrhizas and agrosystem stability. *In* S Gianinazzi, H Schüepp, eds, Impact of Arbuscular Mycorrhizas on Sustainable Agriculture and Natural Ecosystems. Birkhäuser Verlag, Basel, pp 117–131

ge DI, Bowen GD, Skinner MF (1975) Comparative carbohydrate physiology of ecto- and endomycorrhizas. *In* FE Sanders, B Mosse, PB Tinker, eds, Endomycorrhizas. Academic Press, London, pp 149–174

n NS (1991) A critical review of the role of mycorrhizal fungi in the uptake of phosphorus by plants. Plant Soil **134**: 189–208

ert B, Robson A (1994) 65Zn uptake in subterranean clover (*Trifolium*

subterraneum L.) by three vesicular-arbuscular mycorrhizal fungi in a root-free sandy soil. Soil Biol Biochem **26**: 1117–1124

Cox G, Sanders FE, Tinker PB, Wild JA (1975) Ultrastructural evidence relating to host–endophyte transfer in vesicular-abuscular mycorrhizas. *In* FE Sanders, B Mosse, PB Tinker, eds, Endomycorrhizas. Academic Press, London, pp 297–312

Davies FT, Porter JR, Linderman RG (1993) Drought resistance of mycorrhizal pepper plants—independent of leaf phosphorus concentration, response in gas exchange, and water relations. Physiol Plant **87**: 45–53

Doner LW, Bécard G (1991) Solubilization of gellan gels by chelation of cations. Biotechnology Techniques **5**: 25–29

Douds Jr DD, Janke RR, Peters SE. (1993) VAM fungus spore populations and colonization of roots of maize and soybean under conventional and low-input sustainable agriculture. Agric Ecosys Environ **43**: 325–335

Douds Jr DD, Nagahashi G, Abney GD (1996) The differential effects of cell wall-associated phenolics, cell walls, and cytosolic phenolics of host and non-host roots on the growth of two species of AM fungi. New Phytol **133**: 289–294

Eissenstat DM, Graham JH, Syvertsen JP, Drouillard DL (1993) Carbon economy of orange in relation to mycorrhizal colonization and phosphorus status. Ann Bot **71**: 1–10

Galvez L, Douds Jr DD, Wagoner P, Longnecker LR, Drinkwater L, Janke RR (1995) An overwintering cover crop increases inoculum of VAM fungi in agricultural soil. Amer J Alternative Agric **10**: 152–156

Gaspar ML, Pollero RJ, Cabello MN (1994) Triacylglycerol consumption during spore germination of vesicular-arbuscular mycorrhizal fungi. J Am Oil Chem Soc **71**: 449–452

George E, Marschner H, Jakobsen I (1995) Role of arbuscular mycorrhizal fungi in uptake of phosphorus and nitrogen from soil. Crit Rev Biotechnol **15**: 257–270

Gerdemann JW (1968) Vesicular-arbuscular mycorrhizae and plant growth. Ann Rev Phytopathol **6**: 397–418

Gianinazzi-Pearson V, Smith SE, Gianinazzi S, Smith S (1991) Enzymatic studies on the metabolism of vesicular-arbuscular mycorrhizas 5. Is H^+-ATPase a component of ATP-hydrolyzing enzyme-activity in plant–fungus interfaces? New Phytol **117**: 61–74

Giovannetti M, Sbrana C, Logi C (1994) Early processes involved in the host recognition by arbuscules in mycorrhizal fungi. New Phytol **127**: 703–709

Graham JH (1985) Membrane regulation of mycorrhizal symbiosis. *In* Proceedings of the Sixth North American Conference on Mycorrhizae. pp 155–156

Graham JH, Hodge NC, Morton JB (1995) Fatty acid methyl ester profiles for characterization of Glomalean fungi and their endomycorrhizae. Appl Environ Microbiol **61**: 58–66

Harrison MJ (1996) A sugar transporter from *Medicago truncatula*: Altered expression pattern in roots during vesicular-arbuscular (VA) mycorrhizal associations. Plant J **91**: 491–503

Harrison MJ, van Buuren KL (1995) A phosphate transporter from the mycorrhizal fungus *Glomus versiforme*. Nature **378**: 626–629

Hooker JE, Black KE (1995) Arbuscular mycorrhizal fungi as components of

sustainable soil-plant systems. Crit Rev Biotechnol **15**: 201–212

ɟooker JE, Jaizme-Vega M, Atkinson D (1994) Biocontrol of plant pathogens using arbuscular mycorrhizal fungi. *In* S Gianinazzi, H Schüepp, eds, Impact of Arbuscular Mycorrhizas on Sustainable Agriculture and Natural Ecosystems. Birkhäuser Verlag, Basel, pp 191–200

effries P, Barea JM (1994) Biogeochemical cycling and arbuscular mycorrhizas in the sustainability of plant-soil systems. *In* S Gianinazzi, H Schüepp, eds, Impact of Arbuscular Mycorrhizas on Sustainable Agriculture and Natural Ecosystems. Birkhäuser Verlag, Basel, pp 101–115

ennings DH, Burke RM (1990) Compatible solutes—the mycological dimension and their role as physiological buffering agents. New Phytol **116**: 277–283

ɔhansen A, Finlay RD, Olsson PA (1996) Nitrogen metabolism of external hyphae of the arbuscular mycorrhizal fungus *Glomus intraradices*. New Phytol **133**: 705–712

ɔide RT (1991) Nutrient supply, nutrient demand and plant response to mycorrhizal infection. New Phytol **117**:365–386

ɹcey RMN, Paul EA (1982) Carbon flow, photosynthesis, and N_2 fixation in mycorrhizal and nodulated faba beans (*Vicia faba* L.). Soil Biol Biochem **14**: 407–412

ɹrle JE, Pfleger FL (1994) Arbuscular mycorrhizal fungus spore populations respond to conversions between low-input and conventional management practices in a corn-soybean rotation. Agron J **86**: 467–475

ɜel DM, Cooper KM (1978) Incorporation of ^{14}C-labeled substrates by uninfected and VA mycorrhizal roots of onion. New Phytol **83**: 415–426

ɹrtin F, Canet D, Marschal J-P (1985) ^{13}C nuclear magnetic resonance study of mannitol cycle and trehalose synthesis during glucose utilization by the ectomycorrhizal ascomycete *Cenococcum graniforme*. Plant Physiol **77**: 499–502

rtin F, Ramstedt M, Solerhall K, Canet D (1988) Carbohydrate and amino acid metabolism in the ectomycorrhizal ascomycete sphaerosporella brunnea during glucose utilization. Plant Physiol **86**: 935–940

ler RM, Jastrow JD (1992) The role of mycorrhizal fungi in soil conservation. *In* GJ Bethlenfalvay, RG Linderman, eds, Mycorrhizae in Sustainable Agriculture. Agron Soc Amer, Madison, WI, pp 24–44

ɜse B (1959) The regular germination of resting spores and some observations on the growth requirements of an *Endogone* sp. causing vesicular-arbuscular mycorrhiza. Trans Br Mycol Soc **42**: 273–286

ɜse B (1973) Advances in the study of vesicular-arbuscular mycorrhiza. Ann Rev Phytopathol **11**: 171–196

ahashi G, Abney GD, Doner LW (1996a) A comparative study of phenolic acids associated with cell walls and cytoplasmic extracts of host and non-host roots for AM fungi. New Phytol **133**: 281–288

ahashi G, Douds Jr DD (1995) Separation and partial identification of secreted, cell wall–associated, and cytoplasmic-associated phenolic compounds of cultured carrot roots. *In* DL Gustine, HE Flores, Phytochemicals and Health. American Society of Plant Physiologists, Rockville, MD, pp 268–270

ahashi G, Douds Jr DD, Abney GD (1996b) A rapid micro-injection technique

allows for the sensitive detection of root exudate signals which stimulate the branching and growth of germinated VAM fungus spores. Proceedings of the First International Conference on Mycorrhizae. Berkeley, CA, p 91

Peng SB, Eissenstat DM, Graham JH, Williams K, Hodge NC (1993) Growth depression in mycorrhizal citrus at high-phosphorus supply-analysis of carbon costs. Plant Physiol **101**: 1063–1071

Pfeffer PE, Shachar-Hill Y (1996) Plant/microbe interactions. *In* Y Shachar-Hill, PE Pfeffer, eds, Nuclear Magnetic Resonance in Plant Biology. American Society of Plant Physiologists, Rockville, MD, pp 77–107

Pfeffer PE, Shachar-Hill Y, Douds Jr DD, Bécard G (1996) Metabolism of glucose, its uptake and translocation in mycorrhizal leek and transformed carrot roots. *In* Proceedings of the First International Conference on Mycorrhizae. Berkeley, CA, p 96

Ramstedt M, Raida J, Söderhäll K (1987) Metabolism of mannitol in mycorrhizal and non-mycorrhizal fungi. New Phytol **105**: 281–287

Ratcliffe RG (1994) In vivo NMR studies of higher plants and algae. Adv Bot Res **20**: 43–123

Rhodes LH, JW Gerdemann (1975) Phosphate uptake zones of mycorrhizal and non-mycorrhizal onions. New Phytol **75**: 555–561

Ryan MH, Chilvers GA, Dumaresq DC (1994) Colonization of wheat by VA-mycorrhizal fungi was found to be higher on a farm managed in an organic manner than on a conventional neighbor. Plant Soil **160**: 33–40

Saito M (1995) Enzyme activities of the internal hyphae and germinated spores of arbuscular mycorrhizal fungus, *Gigaspora margarita* Becker & Hall. New Phytol **129**: 425–431

Saito M (1997) Regulation of arbuscular mycorrhiza symbiosis: Hyphal growth in host roots and nutrient exchange JARQ **31**: 179–183

Schubert A, Wyss P, Wienken A (1992) Occurrence of trehalose in vesicular-arbuscular mycorrhizal fungi and in mycorrhizal roots. Plant Physiol **140**: 41–45

Shachar-Hill Y, Pfeffer PE, eds (1996) Nuclear Magnetic Resonance in Plant Biology. American Society of Plant Physiologists, Rockville, MD, 260 pp

Shachar-Hill Y, Pfeffer PE, Douds Jr DD, Osman SF, Doner LW, Ratcliffe RG (1995) Partitioning of intermediate carbon metabolism in VAM colonized leek. Plant Physiol **108**: 7–15

Smith SE (1995) Discoveries, discussions and directions in mycorrhizal research. *In* A Varma, B Hock, eds, Mycorrhiza Structure, Function, Molecular Biology and Biotechnology. Springer-Verlag, Berlin, pp 3–24

Smith SE, Read DJ (1997) Mycorrhizal Symbiosis. Academic Press, London, 605 pp

Solaiman MDZ, Saito M (1997) Use of sugars by intraradical hyphae of arbuscular mycorrhizal fungi revealed by radiorespirometry. New Phytol **136**: 533–538

St-Arnaud M, Hamel C, Vimard B, Caron M, Fortin JA (1996) Enhanced hyphal growth and spore production of the arbuscular mycorrhizal fungus *Glomus intraradices* in an in vitro system in the absence of host roots. Mycol Res **100**: 328–332

Tawaraya K, Saito M (1994) Effects of vesicular-arbuscular mycorrhizal infection on amino acid composition in roots of onion and white clover. Soil Sci Plant

Nutr **40**: 339–343

Timmer LW, Leyden RF (1978) Stunting of citrus seedlings in fumigated soils in Texas and its correction by phosphorus fertilization and inoculation with mycorrhizal fungi. J Amer Soc Hort Sci **103**: 533–537

Tisdall JM, Oades JM (1979) Stabilization of soil aggregates by the root systems of ryegrass. Aust J Soil Res **17**: 429–441

Tobar R, Azcon R, Barea JM (1994) Improved nitrogen uptake and transport from ^{15}N-labeled nitrate by external hyphae of arbuscular mycorrhiza under water-stress conditions. New Phytol **126**: 119–122

Villegas J, Williams RD, Nantais L, Archambault J, Fortin JA (1996) Effects of N source on pH and nutrient exchange of extramatrical mycelium in a mycorrhizal Ri T-DNA transformed root system. Mycorrhiza **6**: 247–251

Wang H, Parent S, Gosselin A, DesJardins Y (1993) Vesicular-arbuscular mycorrhizal peat-based substrates enhance symbiosis establishment and growth of three micropropagated species. J Amer Soc Hort Sci **118**: 896–901